



Molecular cloning, expression profiling and functional analyses of a cDNA encoding isopentenyl diphosphate isomerase from *Gossypium barbadense*

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Synopsis

Gossypol, a type of plant defence sesquiterpenoid phytoalexin, is synthesized from the MEP (2C-methyl-D-erythritol 4-phosphate) and MVA (mevalonate) pathway in the isoprenoid biosynthetic system. The key step is the isomerization of IPP (isopentenyl diphosphate) to DMAPP (dimethylallyl diphosphate), which is catalysed by IPI (IPP isomerase; EC 5.3.3.2). A full-length cDNA encoding IPI (designated *GbIPI*) was cloned from *Gossypium barbadense* by RACE (rapid amplification of cDNA ends). The full-length cDNA of *GbIPI* was 1205 bp and contained a 906 bp ORF (open reading frame) encoding a protein of 302 amino acids, with a predicted molecular mass of 34.39 kDa and an isoelectric point of 6.07. Amino acid sequence analysis revealed that the *GbIPI* has a high level of similarity to other IPIs. Southern-blot analysis revealed that *GbIPI* belongs to a small gene family. Expression analysis indicated that *GbIPI* expression is highest in stems, followed by leaves, and is lowest in roots, and that the expression of *GbIPI* could be induced by *Verticillium dahliae* Kleb, MeJA (methyl jasmonate) and SA (salicylic acid). The functional colour assay indicated that *GbIPI* could accelerate the accumulation of β -carotene in *Escherichia coli* transformants. The cloning and functional analysis of *GbIPI* will be useful in increasing understanding of the role of IPI in isoprenoid biosynthesis at the molecular level.

Key words: *Gossypium barbadense*, isopentenyl diphosphate (IPP) isomerase (IPI), isoprenoid biosynthetic pathway, rapid amplification of cDNA ends (RACE), sesquiterpene phytoalexin, *Verticillium dahliae* Kleb

INTRODUCTION

Isoprenoids are found in all living organisms. More than 50 000 isoprenoids with a variety of biological functions have been isolated from micro-organisms, plants and animals. They serve as visual pigments, reproductive hormones, defensive agents, constituents of membranes, components of signal transduction and photoprotective agents [1]. A wide variety of plant terpenoids play an important role in invoking various defence mechanisms and as insect attractants or repellents [2]. Some of the secondary terpenoids have been classified as phytoalexins due

to their potential roles in the natural resistance of plants against phytopathogens [3]. Generally, the defence response in plants is believed to result from an interaction of elicitor molecules with specific receptors in the host, leading to activation of a range of defence-related genes [4]. Terpenoids may be a signal molecule which can generate a defence response.

All terpenoids are biosynthesized by consecutive condensations of IPP (isopentenyl diphosphate) and its isomer DMAPP (dimethylallyl diphosphate) (Figure 1). IPI (IPP isomerase) is a key enzyme and catalyses the interconversion of IPP into DMAPP [5]. IPI is essential in the MVA (mevalonate) pathway, and probably also plays an important role in balancing

Abbreviations used: DMAPP, dimethylallyl diphosphate; FPS, farnesyl diphosphate synthase; IPP, isopentenyl diphosphate; IPI, IPP isomerase; *GbIPI*, *Gossypium barbadense* IPI; LB, Luria–Bertani; MeJA, methyl jasmonate; MEP, 2C-methyl-D-erythritol 4-phosphate; NUP, nested universal primer; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; SA, salicylic acid; UPM, universal primer A mixture; UTR, untranslated region.

The nucleotide sequence data reported will appear in GenBank® EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number DQ979961.

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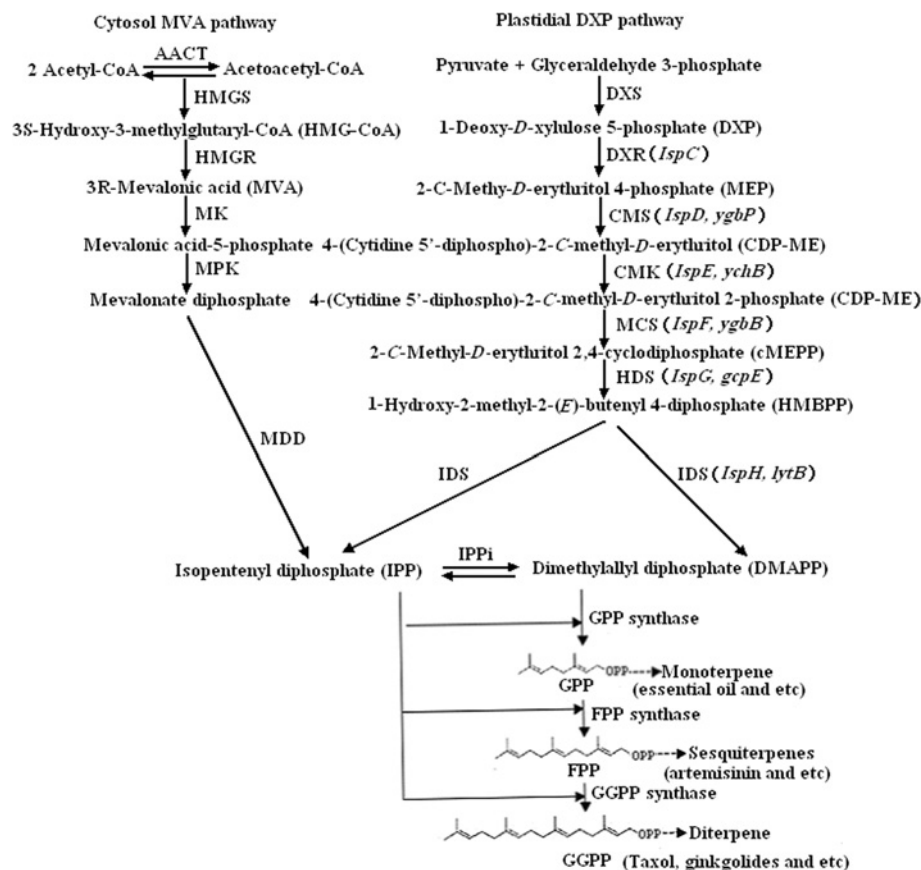


Figure 1 Outline of terpenoid biosynthetic pathway

AACT, acetyl-CoA acyl transferase; CMK, 4-(cytidine 5'-diphospho)-2-C-methylerythritol kinase; CMS, 4-diphosphocytidyl-2-C-methyl-erythritol synthase; DXR, 1-deoxyxylulose-5-phosphate reductoisomerase; DXS, 1-deoxyxylulose-5-phosphate synthase; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl pyrophosphate; HDS, hydroxymethylbutenyl diphosphate synthase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; IDS, diphosphate synthase; IPPi, IPI; MCS, 2-methylcitrate synthase; MVA, mevalonate; MDD, MVA 5-diphosphate decarboxylase; MK, MVA kinase; MPK, MVA phosphate kinase.

the IPP and DMAPP pools in the MEP (2C-methyl-D-erythritol 4-phosphate) pathway [6]. Previous work implied that maize IPI activity was critical in controlling the carotenoid pathway flux [7], forming an influential step in isoprenoid biosynthesis of the prokaryote *Escherichia coli*. It was confirmed further that IPI is a key enzyme in promoting pathway flux [8–10].

In cotton, gossypol is a type of plant defence metabolite (i.e. phytoalexin) that accumulates in specialized secretory glands [11,12]. Owing to the importance of gossypol for the natural resistance of the cotton crop against insects and disease, it is relevant to investigate the isoprenoid biosynthesis pathway in cotton. In previous years, although the IPI cDNAs or genes have been isolated from a array of plant species [13–17], until now there have been no reports on the cloning of *IPI* genes from cotton, and only two reports on the cloning of genes encoding HMGRs (3-hydroxy-3-methylglutaryl CoA reductases) and FPS (farnesyl diphosphate synthase), which are involved in the isoprenoid biosynthesis pathway in cotton [4,12]. In the present study, we report the cloning and characterization of the *IPI* gene

from *Gossypium barbadense* and validate its biological function in *E. coli*. The expression profiles of *GbIPI* (*G. barbadense* IPI) in various cotton tissues and under the induction of *Verticillium dahliae* Kleb, MeJA (methyl jasmonate) and SA (salicylic acid) were also investigated.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of cotton (*G. barbadense*) were sown in pots and pre-germinated at 35°C for 48 h. The germinated plants were then grown in a greenhouse at 27°C with a photoperiod of 14 h light/10 h dark.

Fungal strains and inoculum preparation

The highly aggressive defoliating *V. dahliae* Kleb strain T9, provided by Dr W.Z. Guo (Cotton Research Institute, Nanjing Agricultural University, Nanjing, People's Republic of China),

was used in inoculation experiments. A single fungal spore from the PDA (potato dextrose agar) plate was inoculated into Czapek Broth to incubate for 5–8 days until the concentration of spores reached approx. 10^8 spores/ml. The suspension liquid was adjusted to 10^7 spores/ml with sterile distilled water prior to use [18].

Plant treatment

Cotton leaves (4-weeks-old) were sprayed with 2 mM MeJA or 2 mM SA (both dissolved in 0.1% ethanol and water). At the same time, control leaves were sprayed with only 0.1% ethanol without any elicitors. The treated and control plant leaves were harvested after 0, 6, 12, 24, 36, 48 and 72 h of treatment respectively. Plants (4-weeks-old) were infected with *V. dahliae* Kleb strain T9 by soaking the roots in a suspension of fungal conidia for 10 min at 25°C and then returned to their original pots. The control roots were soaked with sterile distilled water for 10 min. The treated and control plant roots were harvested after 0, 3, 6, 12, 24, 36, 48 and 72 h respectively. All of the control and treatment experiments were carried out in triplicate. The plant leaves and roots were subsequently harvested for RNA extraction.

RNA and DNA isolation

Different tissues of *G. barbadense*, including roots, stems and leaves (treated and untreated controls), were excised and pulverized in liquid nitrogen and the total RNA and genomic DNA were extracted using a CTAB (cetyltrimethylammonium bromide) method with modifications [19,20]. The quality and concentration of the extracted RNA and DNA were checked by agarose-gel electrophoresis (1% gels) and quantified by a nucleic acid protein analyser (DU-640, Beckman). The RNA and DNA samples were stored at -70°C and -20°C respectively prior to use.

Cloning of 3'-RACE (rapid amplification of cDNA ends), 5'-RACE and full-length cDNA of *GbIPI*

cDNA synthesis was carried out by using the SMARTTM RACE amplification kit (Clontech). Single-stranded cDNA was reverse transcribed from 5 µg of total RNA with an oligo(dT) primer according to the manufacturer's instructions (PowerScriptTM, Clontech). After RNaseH treatment, the single-stranded cDNA mixtures were used as templates for PCR. Two degenerate oligonucleotide primers, GbipiF [5'-CG(A/C/T)-CT(C/T)ATGTT(C/T)GA(C/G/A)GA(T/C)GAATG(C/T)AT-3'] and GbipiR [5'-CCACCA(T/C)TT(G/C)A(A/T)CAA(A/G)AA-(A/G)TT(A/G)TC-3'], were designed and synthesized based on highly conserved regions of amino acid sequence between several plant IPIs and were used for the amplification of the core fragment of *GbIPI* by standard gradient PCR from 51°C to 65°C. The core fragment was amplified at 58°C and then subcloned into the pMD18-T vector (TaKaRa), transformed into *E. coli* DH5α cells and then sequenced using the ABI 3730 sequencer (Applied Biosystems). A BLASTN search confirmed that it was highly homologous with other plant IPI genes. Subsequently, the

core fragment was used to design and synthesize gene-specific primers to clone the full-length cDNA by RACE.

The 3'- and 5'-ends of the *GbIPI* cDNA were obtained using the SMARTTM RACE cDNA amplification kit (Clontech). Two 3'-gene-specific primers were designed for 3'-RACE amplification. For the first round of amplification of the 3'-end of *GbIPI* cDNA, Gbipi3-1 (5'-GGTGAGGAATGCTGCCCAA-AGGAAG-3') and UPM (universal primer A mixture) long: 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' and short: 5'-CTAATACGACTCACTATAGG-GC-3') were used as PCR primers, with 3'-RACE-ready cDNA used as the template. For nested PCR of 3'-RACE, Gbipi3-2 (5'-TGATTACCTCCTATTTCATTGTCCG-3') and NUP (nested universal primer) (5'-AAGCAGTGGTATCAACGCAGAGT-3', provided by the SMARTTM RACE cDNA amplification kit) were used as PCR primers with the first round PCR products used as templates. Two 5'-gene-specific primers were designed for 5'-RACE amplification. For the first cycle of amplification of the 5'-end of *GbIPI* cDNA, Gbipi5-1 (5'-CCTCTGCTACCT-CATCAGGGTTTGG-3') and UPM were used as the primers, with 5'-RACE-ready cDNA used as the template. For nested PCR amplification of 5'-RACE, Gbipi5-2 (5'-CTTCTTTGGG-CAGCATTCTCACC-3') and NUP were used as primers, with the first round PCR products used as templates. An Advantage 2 PCR Kit (Clontech) was used in the first round and nested PCR amplification of the 5'- and 3'-ends of *GbIPI* cDNA, and the procedures were carried out according to the manufacturer's protocols (SMARTTM RACE cDNA amplification kit, Clontech): 25 cycles of amplification (30 s at 94°C, 30 s at 68°C and 1 min at 72°C). Subsequently the 3'- and 5'-RACE products were subcloned into the pMD18-T vector, followed by DNA sequencing. By aligning and assembling the sequences of the 3'-RACE and 5'-RACE products and the core fragment using Contig Express (Vector NTI Suite 8.0), the fulllength cDNA sequence of *GbIPI* was deduced and subsequently amplified by PCR using the primers FGbipiF (5'-GAGAGTAGAGCATATGCAGAGAGCAGCAGACAGACC-3') and FGbipiR (5'-CTGGAAGTACCCATTGACAACTTTGTTC-ATGTC-3') with 3'-RACE-ready cDNA as the template under the following conditions: 3 min at 94°C, followed by 35 cycles of amplification (45 s at 94°C, 45 s at 60°C and 90 s at 72°C) and 7 min at 72°C. The PCR product was purified and cloned into the pMD18-T vector, followed by DNA sequencing. Three independent positive clones were picked out and sequenced to avoid PCR errors.

Sequence analysis

BLAST analysis, the ORF (open reading frame) finder and molecular-mass calculation of the predicted protein were carried out online (<http://www.ncbi.nlm.nih.gov> and <http://cn.expasy.org>). Vector NTI Suite 8 (InforMax) was used for multiple alignment analysis of the full-length IPI amino acid sequences.

Southern-blot analysis

Aliquots of DNA (40 µg/sample) were digested overnight with BglII, EcoRI, and XbaI respectively, separated by agarose-gel

electrophoresis (1% gels) and transferred on to Hybond-N⁺ nylon membrane (Amersham Biosciences) by capillary blotting. The 654 bp probe was generated by PCR using the full-length cDNA of *GbIPI* as a template with primers RTGbipiF (5'-CATACGCCCTCTCACTTTCTCGCTC-3') and RTGbipiR (5'-CAATGAATAGGAGGTAATCAAGTT-3'). In the full-length cDNA of *GbIPI*, there are no BglII, EcoRI and XbaI digestion sites. Probe labelling (biotin) and signal detection were performed using the Amersham Multiprime Labelling system and Gene Images CDP-Star detection procedures.

Expression profile analyses

One-step RT-PCR (reverse transcription-PCR) was carried out to investigate the expression profiles of *GbIPI* in different tissues, including roots, stems and leaves, and under different elicitor treatments, including SA (2 mM), MeJA (2 mM) and *V. dahliae* *Kleb* respectively. Aliquots of total RNA (500 ng/sample) were used as templates for one-step RT-PCR with the forward primer RTGbipiF and the reverse primer RTGbipiR, specific for the coding sequence *GbIPI* using the One-Step RNA PCR kit (TaKaRa). The reaction was performed by reverse transcription at 50°C for 50 min, and then denaturation at 94°C for 2 min, followed by 25 cycles of amplification (94°C for 30 s, 55°C for 30 s and 72°C for 1 min). Meanwhile, an internal control using the specific primers U1 (5'-AAGACCTACACCAAGCCAA-3') and U2 (5'-AAGTGAGCCCACACTTACCA-3') [21] designed to amplify the housekeeping gene ubiquitin (GenBank® nucleotide sequence database accession number AY189972), was also performed under the following conditions: 50°C for 50 min and 94°C for 2 min, followed by 25 cycles of amplification (94°C for 30 s, 55°C for 30 s and 72°C for 1 min). The amplified products were separated by agarose-gel electrophoresis (1% gels) and analysed with a gene analysis software package (Gene Company).

Functional assay of *GbIPI* in *E. coli* TOP10 F' strain

The plasmids pTrc-AtIPI and pAC-BETA, kindly provided by Dr Francis X. Cunningham Jr (Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, U.S.A.), were used to test the biological function of *GbIPI*. The plasmid pAC-BETA contains all of the genes required for the synthesis of β -carotene, including *crtE* [GGPP (geranylgeranyl pyrophosphate) synthase], *crtB* (phytoene synthase), *crtI* (phytoene desaturase) and *crtY* (lycopene cyclase) [22], and also retains a chloramphenicol resistance gene. The plasmid pTrc-AtIPI retains an ampicillin resistance gene and an *AtIPI* gene whose product can accelerate the accumulation of β -carotene [15]. The *E. coli* TOP10 F' strain was used as a host to test the function of *GbIPI*. The experimental procedure was performed as follows: a fragment containing the coding region of *GbIPI* was amplified by PCR using ORFGbipiF (5'-ATGCTTATGCTTTTAAATACTCC-3') and ORFGbipiR (5'-TCA-CAGCTTATGAATGGTTTCCATGTCAC-3'). The PCR product was purified and cloned into the pMD18-T vector and DNA sequenced. Subsequently, a pair of primers containing BglII and NotI digestion sites were designed and synthesized to amp-

lify the ORF region. Both the fragment and the plasmid pTrc-AtIPI were digested with BglII and NotI. The coding region of *GbIPI* was then cloned into the expression vector pTrc to obtain the plasmid pTrc-GbIPI, and was subsequently sequenced. The plasmid pTrc-AtIPI was digested by PstI and ligated with T4 ligase as control. The plasmids pTrc-GbIPI and pAC-BETA were used to co-transform *E. coli* TOP10 F' cells, whereas pAC-BETA and an empty plasmid (pTrc) were co-transformed and a single plasmid pAC-BETA was transformed into *E. coli* TOP10 F' as controls. Transformants were selected on solid LB (Luria-Bertani) medium containing ampicillin (150 mg/l) and chloramphenicol (50 mg/l) at 37°C for 48 h.

Quantification of β -carotene accumulated in *E. coli*

The *E. coli* TOP10 F' cells, transformed with the plasmids pTrc-GbIPI and pAC-BETA and the control plasmids pTrc and pAC-BETA respectively, were grown at 37°C in LB liquid medium containing 150 mg/l ampicillin and 50 mg/l chloramphenicol to an attenuation (*D*) at 600 nm of 1.0. Aliquots of *E. coli* cells were harvested by centrifugation at 13 000 *g* for 3 min and washed once with sterile water, followed by recentrifugation. The cells were resuspended in acetone (1 ml) and incubated at 55°C for 15 min in the dark. The samples were centrifuged again at 13 000 *g* for 10 min, and the acetone supernatant containing β -carotene was transferred into a clean tube. The β -carotene content of the extracts was quantified by measuring the optical absorbance (*A*) at 474 nm using a PowerWave XS Microplate Spectrophotometer (BioTek) and comparing it with authentic β -carotene (Sigma) [23]. Results are means for three independent determinations.

RESULTS

Molecular cloning of the full-length cDNA of *GbIPI*

On the basis of the high level of conservation in the sequence of plant IPI genes, we designed two degenerate oligonucleotide primers (GbipiF and GbipiR) to amplify the core cDNA fragment of *IPI* from *G. barbadense*. A 597 bp PCR product was obtained, ligated into the pMD18-T vector and DNA sequenced. A BLASTN search of the sequence revealed that the obtained fragment has a high level of homology with *IPI* genes from other plant species. Thus gene-specific primers were designed for the amplification of the 5'-end and 3'-end cDNA fragments of *GbIPI* based on the 597 bp core sequence obtained. By 5'- and 3'-RACE, cDNA fragments of 582 bp and 510 bp were amplified respectively. The 5'-end, 3'-end and the core fragment obtained were then aligned and assembled using Vector NTI Suite 8.0, and the full-length cDNA sequence was deduced and subsequently confirmed by DNA sequencing. The full-length cDNA is 1205 bp in length, with a 906 bp ORF encoding 302 amino acids, with a 3'-UTR (untranslated region) of 260 bp downstream from the stop codon and a 5'-UTR of 39 bp upstream of the first ATG codon. The *GbIPI* sequence has been deposited into GenBank and will be designated as DQ979961.

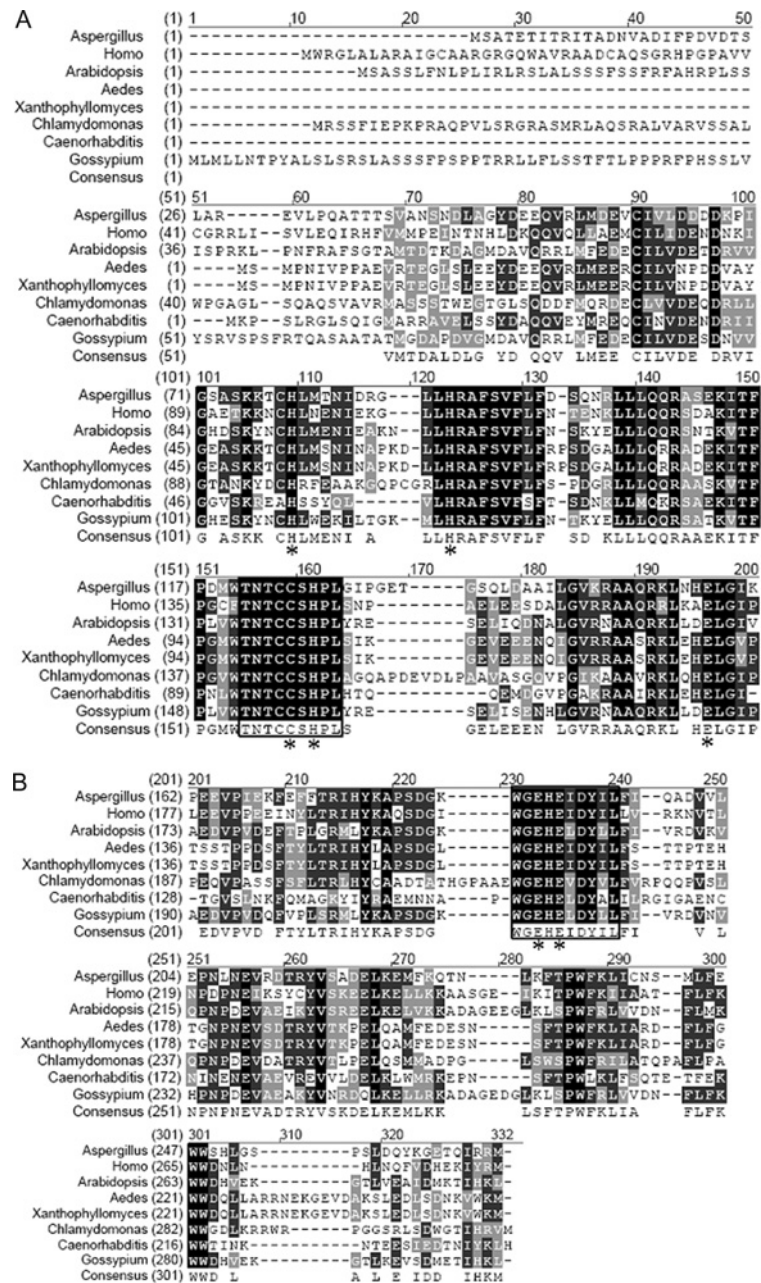


Figure 2 Alignment of the deduced amino acid sequences of GbIPI and other IPIs

Aspergillus nidulans (GenBank® Entrez Protein database accession number AA085433), *Homo sapiens* (NP_004499), *Arabidopsis thaliana* (NP_186927), *Aedes aegypti* (ABF18503), *Xanthophyllomyces dendrorhous* (BAA33979), *Chlamydomonas reinhardtii* (AAC32601), *Caenorhabditis elegans* (AAT08468) and *Gossypium barbadense* IPIs are aligned. The identical amino acids are indicated by black shading, and conserved amino acid residues are indicated by dark grey shading. Blocks of similar amino acids are indicated by light grey shading. Residues known to be critical for the catalytic activity of the enzyme are indicated with asterisks (*). A conserved cysteine motif and a conserved glutamate motif are shown in boxes.

Bioinformatic analysis of GbIPI

The GbIPI protein was predicted to have a molecular mass of 34.39 kDa and a theoretical pI of 6.07 (<http://cn.expasy.org/tools/protparam.html>). Protein-protein BLAST analysis showed that

the deduced GbIPI sequence has high homology with IPI sequences from other plants, including those from *Ipomoea batatas* (74% identity, 84% similarity), *Tagetes erecta* (87% identity, 93% similarity), *Hevea brasiliensis* (85% identity, 91% similarity),

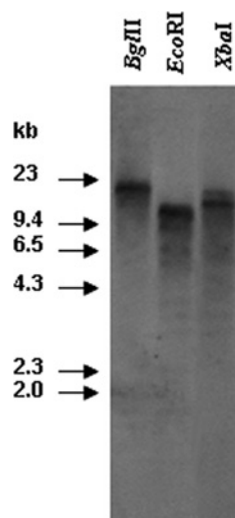


Figure 3 Southern-blot analysis of *GbIPI*

Genomic DNA isolated from the leaves of *G. barbadense* was digested with BglII (lane 1), EcoRI (lane 2) and XbaI (lane 3) respectively, followed by hybridization with a 654 bp fragment generated by PCR (used as the probe). DNA fragment sizes are indicated on the left-hand side (in kb).

Nicotiana tabacum (86% identity, 93% similarity) and *Oryza sativa* (87% identity, 92% similarity), suggesting that *GbIPI* belongs to the IPI family.

It has been known that IPIs contain a number of highly conserved regions that are common from higher plants and humans to yeasts. Multiple-alignment analysis shows that these highly conserved residues containing three histidines (His²⁵, His³² and His⁶⁹), two glutamate residues (Glu¹¹⁴ and Glu¹¹⁶), Cys⁶⁷ and Glu⁸⁷, also exist in *GbIPI* [(*)], see Figure 2].

Southern-blot analysis

Southern-blot analysis was carried out by digesting the genomic DNA of *G. barbadense* with BglII, EcoRI and XbaI respectively, followed by hybridization with a 654 bp probe generated by PCR. As shown in Figure 3, more than one hybridizing band was present in the second and third lanes, indicating that *GbIPI* belonged to a small gene family.

Semi-quantitative RT-PCR analyses of *GbIPI*

Semi-quantitative RT-PCR was used to investigate the expression profiles of *GbIPI* in different tissues of *G. barbadense* using the primers RTGbipiF and RTGbipiR (see above). Total RNA was extracted from roots, stems and leaves respectively. The result showed that *GbIPI* is constitutively expressed in all of the tissues tested, but is highest in stems, followed by leaves, and is lowest in roots (Figure 4).

The effect of various elicitors on *GbIPI* expression patterns was analysed by one-step RT-PCR. Total RNA of *G. barbadense* was used as a template to detect the transcript levels at different time points after each elicitor treatment. The result showed that the expression of *GbIPI* in roots was influenced by *V. dahliae* Kleb strain T9 treatment. *GbIPI* expression was increased to a

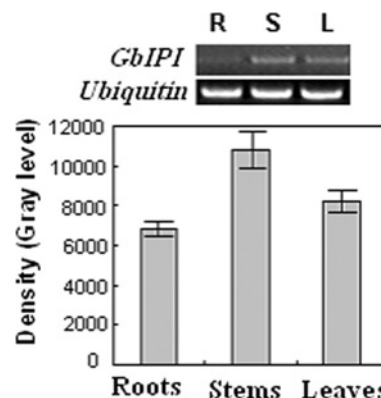


Figure 4 Expression profiles of *GbIPI* in various tissues of *G. barbadense*

Total RNA was isolated from roots (R), stems (S) and leaves (L) respectively, and subjected to semi-quantitative one-step RT-PCR analysis. The ubiquitin gene (GenBank® nucleotide database accession number AY189972) was used as a control to normalize the amount of RNA loaded in each PCR. Results are means $\pm$ S.D. ($n = 3$).

maximum level at 24 h after treatment with *V. dahliae* Kleb, then declined thereafter (Figure 5A), suggesting that *GbIPI* may be involved in the disease response of cotton. The expression of *GbIPI* was also influenced by MeJA and SA treatments. Under MeJA treatment, the expression of *GbIPI* was rapidly increased in the first 6 h, and reached a maximum level after 12 h, and then declined (Figure 5B). In a similar manner, the expression of *GbIPI* was also influenced by SA treatment, reaching a maximum level at 24 h after treatment and declining thereafter (Figure 5C).

GbIPI increased β -carotene production in *E. coli* TOP10 F' cells

To identify whether *GbIPI* encoded a functional protein, the *E. coli* TOP10 F' strain was co-transformed with the plasmids pAC-BETA and pTrc-*GbIPI* harbouring the *GbIPI* coding region. The *E. coli* TOP10 F' strain was also co-transformed with pAC-BETA and the empty plasmid pTrc as a control. The result showed that *E. coli* TOP10 F' containing pTrc-*GbIPI* and pAC-BETA accumulated significantly higher β -carotene levels compared with the control (Figure 6). The amount of β -carotene produced by the individual strains was quantified in liquid culture. The final β -carotene content of recombinant *E. coli* strains containing pTrc-*GbIPI* and pAC-BETA was 3.19 mg/l and approx. three times that of the control (1.05 mg/l). All these results demonstrated that *GbIPI* encodes a functional protein and plays an important role in promoting carotenoid pathway flux.

DISCUSSION

Many genes that encode IPI have been cloned from higher plants. However, there are no reports on the cloning of IPI genes from cotton. In the present study, the full-length cDNA encoding IPI was cloned from *G. barbadense*. Sequence analysis showed that

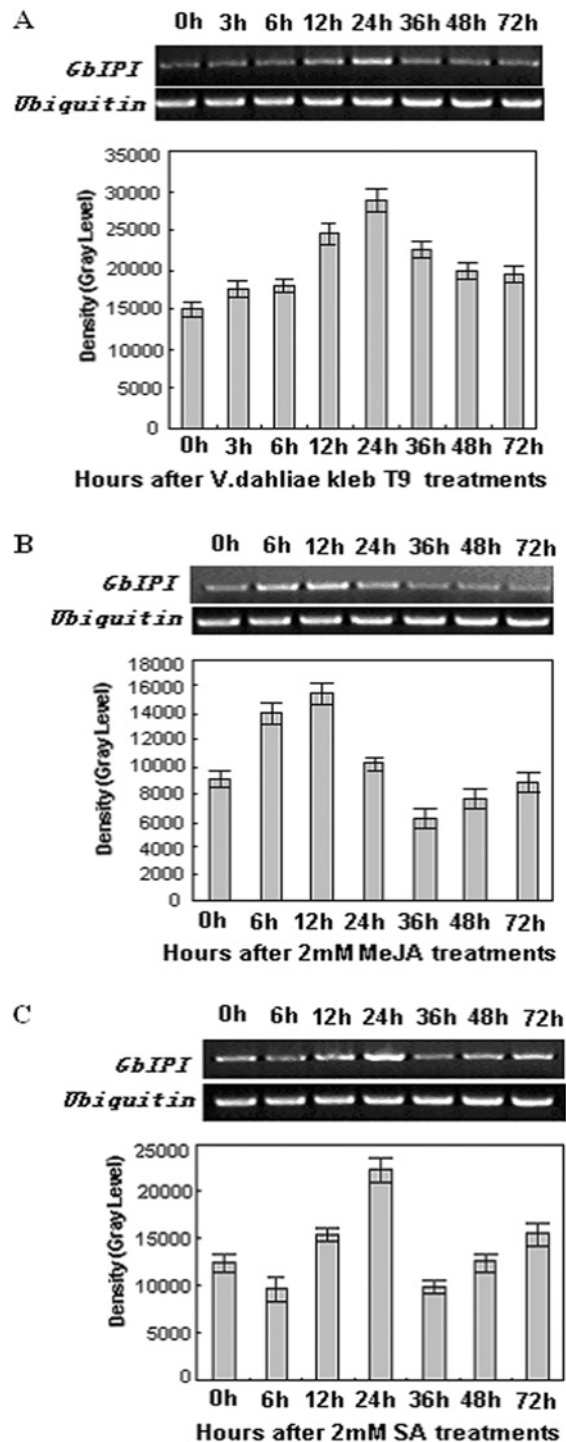


Figure 5 Expression profiles of *GbIPI* under various treatments including *V. dahliae* Kleb T9 infection (A), 2 mM MeJA (B) and 2 mM SA (C)

(A) Total RNA was isolated from roots of *G. barbadense* at 0, 3, 6, 12, 24, 36, 48 and 72 h after *V. dahliae* Kleb T9 infection. (B,C) Total RNA was isolated from leaves at 0, 6, 12, 24, 36, 48 and 72 h after MeJA and SA treatment respectively. All samples were analysed by one-step RT-PCR and the ubiquitin gene was used as a control to normalize the amount of RNA loaded in each PCR. All results are means $\pm$ S.D. ($n=3$)

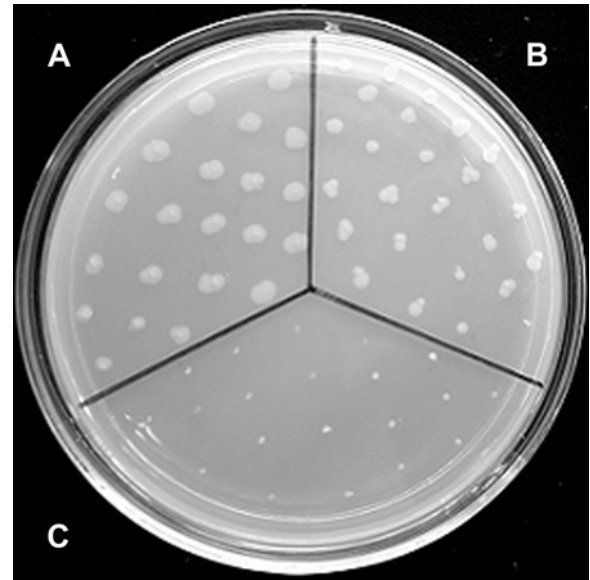


Figure 6 Functional demonstration of *GbIPI* activity using *E. coli* TOP10 F' strain

E. coli cells were transformed with pAC-BETA and pTrc-*GbIPI* harbouring the *GbIPI* gene (A), pAC-BETA and pTrc (empty vector) (B) or pAC-BETA (C).

GbIPI had a high homology with other IPIs, with the conserved catalytic activity amino acids present in the counterparts from other higher plants, animals, human, fungi, yeasts and eelworm (Figure 2).

It has been shown previously that there are two types of IPI [24,25]. Type I IPI contains a conserved cysteine residue in a TNTCCSHPL motif and a conserved glutamate residue in a WGEHXYDY motif [6]. Type II IPI has a conserved motif, including three glycine-rich sequences, MTGG, GXGGT and (A/G)SGG [24]. These highly conserved residues have been shown to be critical for the catalytic activity of the enzyme [26–29]. Multiple alignment analysis showed that *GbIPI* contains the conserved cysteine residue in the TNTCCSHPL motif and conserved glutamate residue in the WGEHXYDY motif (Figure 2), suggesting that *GbIPI* belongs to the type I IPI family.

In the plant species studied to date, IPI is encoded by a small gene family of two genes in *Arabidopsis thaliana* [14], *Hevea brasiliensis* [5], *Adonis aestivalis* [15], *Lactuca sativ* [15], *Nicotiana tabacum* [16] and *Melaleuca alternifolia* [17], or by a single gene in *Oryza sativa* and *Tagetes erecta* [15]. Southern-blot analysis indicated that the *GbIPI* gene also belonged to a small gene family (Figure 3), consistent with the results from other plant IPIs.

MeJA and SA play key roles in regulating plant responses to various stresses [30]. In the present study, we demonstrated that the expression of *GbIPI* was characteristically induced by SA, MeJA and *V. dahliae* treatments, suggesting that the *GbIPI* gene is induced by certain components of the defence/stress signalling pathways. Induction of IPI with elicitor-treated cotton might be satisfied by the requirement of the balance of IPP–DMAPP as a

substrate for terpene biosynthesis. For instance, gossypol, a sesquiterpene phytoalexin, confers natural resistance against various insects and diseases, as showed in a previous study using cultured *N. tabacum* cells [3].

Carotenoids are synthesized in plants. *E. coli* cannot synthesize carotenoids, as they do not possess carotenogenic genes. But *E. coli* cells harbouring the plasmid pAC-BETA have the ability to accumulate β -carotene [31], which can be used as a visible marker, providing an easily screenable phenotype, which has been widely employed to evaluate the roles of genes involved in the carotenoid biosynthesis pathway [8,15,17,32–34]. The recombinant *E. coli* strains carrying the exogenous *GbIPI* gene accumulated elevated levels of β -carotene compared with the control strains, thus demonstrating that *GbIPI* encodes a functional protein and plays an important role in promoting carotenoid pathway flux.

In summary, we have successfully cloned and characterized the first functional gene encoding IPI, a committed-step enzyme involved in isoprenoid biosynthesis, from *G. barbadense*. The cloning and characterization of *GbIPI* will be helpful to explain more about the role of IPI in terpenoid biosynthesis and will facilitate further study of IPI with regard to the mechanism of the defence response of cotton at the molecular level.

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